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ORIGINAL ARTICLE

The use of nails as an alternative matrix for the long-term detection of previous drug intake: validation of sensitive UHPLC-MS/MS methods for the quantification of 76 substances and comparison of analytical results for drugs in nail and hair samples

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Abstract
Purpose This article describes the validation of multi-target methods for the determination of 76 different analytes in hair and nail samples. Segmented hair and nail samples taken from autopsy cases were included in this study.
Method Drugs of abuse, psychotropic drugs, and other drugs were included for the validation of this method. Hair and nail samples were ground using a ball mill and extracted for 18 h. Extracts were measured using a UHPLC-triple quadrupole-mass analyzer. Analytes were separated on a RP 18 column under gradient elution of the mobile phases, water with 0.1 % formic acid and acetonitrile. Whole nail and hair samples from seven autopsy cases were split into segments and analyzed.
Results The entire method was validated according to the German Society of Forensic Toxicology guidelines. In addition, the concentration ratios of selected substances and their metabolites were calculated. Similar concentration ratios in hair and nails were detected for 3,4-methylendioxyamphetamine (MDA)/3,4-methylenedioxy-*N*-methylamphetamine (MDMA), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)/methadone, and bisnortilidine/nortilidine in some of the cases. Reduced 6-monoacetylmorphine (6-MAM) and cocaine concentrations were observed as a result of sample preparation using the ball mill. Previous heroin intake could be assumed from the detection of 6-MAM and morphine in only one nail segment in one case.
Conclusions Nail samples may serve as an alternative matrix for the detection of long-term consumption of a wide range of drugs. Based on our results, drug concentrations in nails are not comparable to those in hair. The main mechanisms for drug incorporation into the nails may be during the formation of the nail plate by the germinal matrix. However, external contamination can also affect the analysis of nail clippings.

Keywords Drugs of abuse · Psychotropic drugs · Hair analysis · Nail analysis · Postmortem samples · Segmental analysis

Introduction

Hair analysis is recognized as a retrospective technique to detect chronic exposure to drugs, or intake of drugs several months prior to sampling, due to the stable drug incorporation into hair samples over several months [1–6]. This can be used for the identification of current drug use and/or addiction, or exposure to drugs in the past [7]. The concentration of drugs in hair samples can give an overview of the level of previous consumption by an individual, and can be used to detect variation between low consumption levels and levels found in regular consumers [8]. The segmentation of hair samples can be used to detect as little as one previous drug use [9–12]. Destructive cosmetic hair procedures, including dyeing or bleaching, can damage the structure of hair, which provides routes of contamination and the loss of incorporated drugs [13–15]. Moreover, hair is subjected to exposure to sunlight, heat, and water, which could decrease the concentration of drugs in hair samples.

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Hair and nails are both types of keratinized matrices with the potential to accumulate xenobiotics [16–18]. In some postmortem cases there is no hair available for testing, due to states of putrefaction, limited hair growth over a lifetime, short hairstyles or age-related limited hair growth. Nails provide a potential alternative matrix for testing to provide information for medical psychological assessment to prove drug abstinence. The advantage of using nail matrix for detection purposes is the greater availability of samples in men compared to hair samples, as many men have short hair. These samples can be used when these individuals need to prove their abstinence in order to regain their driver license. In these cases, an alternative matrix is required for the detection of previous or chronic consumption of drugs. Although drug incorporation is known to take place via blood capillaries, sweat and sebum, and external contamination of hair has been described previously, there is limited information about the mechanisms of drug incorporation into the nail matrix [1, 19]. Previous reports have described the detection of drugs in nail samples after only one intake of a specific drug [20, 21]. In addition, it has also previously been shown that a wide range of different analytes may be detected in hair and nail samples taken at postmortem [22].

In contrast to hair, nail formation does not exclusively occur from the root. The growth of nails occurs in two different areas, 80 % of the nail matrix originates from the nail root and 20 % is produced from the nail bed during progressive growth [23, 24]. The growth rate of nails has been reported to range between 1.9 and 4.4 mm/month (mean 3.47 mm/month) for fingernails and between 0.9 and 2.1 mm/month (mean 1.62 mm/month) for toenails [25]. The incorporation of drugs into nails may occur via sweat and external contamination from the handling of powders, liquids, and tablets. Therefore, nail analysis may be preferred in cases where the hair has been treated with destructive hair cosmetic products and procedures (e.g., hair bleaching and dyeing).

The objective of the present study was to investigate the incorporation of drugs into nails and the comparison of drug concentrations in hair and nail samples from postmortem cases with a promising case history (e.g., drug addicts and individuals who suffered from depressive disorders).

Moreover, further details of the windows of detection related to different nail segments are still required [23].

Validated methods, based on international guidelines, are required for the analysis of drug concentrations in nail samples. It may be possible to gain more information about drug incorporation into nails (e.g., via sweat, blood or the germinal matrix during nail growth) from the segmentation of whole nail samples from postmortem cases.

Materials and methods

Reagents and chemicals

Solvents used for sample preparation were of analytical grade and obtained from Merck KGaA (Darmstadt, Germany). The mobile phase LC-MS grade acetonitrile was obtained from Fisher Scientific GmbH (Schwerte, Germany) and water and formic acid (>99 % purity) were from Acros Organics (Geel, Belgium).

Deuterated standards of 6-monoacetylmorphine (6-MAM)-d3, alpha-hydroxy-alprazolam-d5, alprazolam-d5, 7-aminoflunitrazepam-d7, amphetamine-d5, benzoylecgonine-d5, buprenorphin-d4, clonazepam-d4, cocaine-d3, cocaethylene-d3, codeine-d3, diazepam-d5, dihydrocodeine-d3, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)-d3, flunitrazepam-d7, fentanyl-d5, ketamine-d4, lorazepam-d4, methamphetamine-d5, 3,4-methylenedioxyamphetamine-d5, 3,4-methylenedioxy-*N*-ethylamphetamine (MDEA)-d6, 3,4-methylenedioxy-*N*-methylamphetamine (MDMA)-d5, methadone-d9, methylecgonine-d3, morphine-d3, nordazepam-d5, norbuprenorphine-d3, norcocaine-d3, nortilidine-d3, oxazepam-d5, oxycodone-d3, tilidine-d6, temazepam-d5, and tramadol-d3 were purchased from LGC Standards GmbH (Wesel, Germany) and each had a purity of 99 %. A deuterated standard mixture containing 1 ng/ml in methanol was compounded in the laboratory. This mixture was added to each nail and hair sample.

An undeuterated standard mixture of 3-hydroxy-bromazepam, 6-monoacetylmorphine (6-MAM), 7-aminoflunitrazepam, alpha-hydroxy-alprazolam, 9-hydroxy-risperidone, acetylcodeine, amisulpride, amphetamine, benzoyecgonine, bisnortilidine, buprenorphine, bupropion, chlorpromazine, citalopram, clomipramine, clonazepam, clozapine, cocaetyhlene, cocaine, codeine, desmethyldoxepin, diazepam, dihydrocodeine, diphenhydramine, doxepin, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), fentanyl, flunitrazepam, fluphenazine, fluvoxamine, fluoxetine, gabapentin, haloperidol, heroin, ketamine, lorazepam, 3,4-methylendioxyamphetamine (MDA), 3,4-methylenedioxy-*N*-ethylamphetamine (MDEA), 3,4-methylenedioxy-*N*-methylamphetamine (MDMA), maprotiline, methadone, methamphetamine, methylecgonine, metoprolol, mianserin, midazolam, mirtazapine, melperone, methylphenidate, morphine, *N*-desmethylcitalopram, *N*-desmethylclomipramine, norbuprenorphine, norcocaine, nordazepam, norfentanyl, nortilidine, nortriptyline, norclozapine, opipramol, olanzapine, oxazepam, oxycodone, paroxetine, promethazine, quetiapine, sertraline, sulpiride, risperidone, temazepam, tilidine, tramadol, trimipramine, tranylcypromine, venlafaxine, and zolpidem were added to the calibration samples.

Instruments and software

A LC-QQQ-MS (LC infinity 1290 with binary pump and degasser and 6460 Triple Quadrupole (QQQ), Agilent Technologies, Waldbronn, Germany) was used for sample analyses. Liquid chromatography was performed on a Kinetex C18 column (2.1 × 150 mm, 1.7 µm, Phenomenex, USA). For the measurement, an electrospray-ionization (ESI) Agilent Jet Stream source operated in positive ionization mode and dynamic multiple reaction monitoring (dMRM) were used. For the method validation and sample measurements, the data were analyzed using MassHunter Workstation Software version B.06.00, Agilent MassHunter Optimizer and Valistat 2.0. The quantification was performed using MassHunter Quantitative Analysis (B.06.00).

Blank hair and nail samples

Method validation was performed using blank hair samples obtained from volunteers. Blank nail clippings from toenails and fingernails were also collected from volunteers for the negative pool. The matrix effects were analyzed using five different hair and nail samples collected from volunteers.

Sample collection

Samples from postmortem cases were collected at the beginning of each autopsy to avoid contamination with body fluids. Hair samples were cut as close as possible to the scalp from the vertex region of the head. The lock of hair obtained was approximately the thickness of a pencil. The hair samples were bundled at the proximal end. Whole nails ($n = 5$) were only obtained from bodies that were already in a state of putrefaction. If other bodies the free edge of the fingernails and toenails (1–3 mm; $n = 2$) was cut using nail scissors. The samples were stored under dark and dry conditions at room temperature until analysis.

Segmentation of hair and nail samples

The segmentation of the nail and hair matrices was performed for each of the cases where whole nail samples were available. Figure 1 shows the segmentation of hair and nail samples in detail. The hair and nail samples were divided into different segments that matched the same time windows. Different growth rates of finger- and toenails were considered. A whole nail sample was divided into four different segments (Fig. 1): the nail clipping (A), whole length of nail (B), whole length of nail plate but without the bottom of nail plate that was in constant contact with the nail bed, and the whole length of nail plate (D). The parts C and D were divided into different segments (1, 2, 3, …) that matched the same window as the hair segments (1, 2, 3, …).

Hair and nail sample preparation

For analysis, 20 mg of hair and nail samples were cut into small pieces and washed, firstly with water (1 × 10 ml wash) then with acetone (2 × 10 ml washes) to minimize external contamination. The samples were dried overnight at room temperature. The cleaned and dried samples were transferred into 1.5 ml Eppendorf tubes and were ground using a ball mill (Retsch, Haan, Germany) at 30.0 s^{-1} with six balls (Ø = 2 mm) for 10 min for hair samples and 15 min for nail samples. After this, 500 µl of extraction buffer (methanol/acetonitrile/H_2O with ammonium formate; 25/25/50) and 5 µl of the internal standard mix (1 ng/µl) were added. The samples were shaken at 800 rpm for 18 h at 40 °C. The supernatant from the first extraction was transferred into another vial and stored, while for the second extraction another 500 µl of extraction buffer were added to the powder, which was shaken at 800 rpm for another 18 h at 40 °C [22, 26]. Both the supernatants were combined and then filtered through a RC membrane filter (Ø = 4 mm, 0.2 µm pore size, Phenomenex) into a final vial for measurement.

Characteristics of chromatography, internal standards, transitions, and retention times

For the detection of each of the 76 different substances in the hair and nail samples, two different methods were validated. By gradient elution [mobile phase water with 0.1 % formic acid (eluent A) and acetonitrile (eluent B)], an evenly spread elution over a runtime of 11 min was obtained for the psychotropic drugs, and a runtime of 16 min was obtained for the drugs of abuse and other drugs of forensic interest. The first method included a mobile phase gradient: 0–1 min, 10 % B; 1–2 min, 22 % B; 2–5 min, 30 % B; 5–7 min, 34.8 % B; 7–8.5 min, 39 % B; and from 8.5–10 min there was a linear decrease to 10 % B (starting conditions).

The second method for the detection of drugs of abuse and other drugs of forensic interest included the same mobile phases with the following gradient: 0–1 min, 5 % B; 1–5 min, 20 % B; 5–14 min 65 % B; and 14–16 min, 95 % B. For both methods the injection volume was 5 µl and the flow rate was 0.4 ml/min. The temperature of the column was set at 40 °C. Source parameters had a drying gas (nitrogen) temperature of 210 °C, drying gas flow of 10 L/min, nebulizer pressure of 45 psi and a capillary voltage of 3000 V.

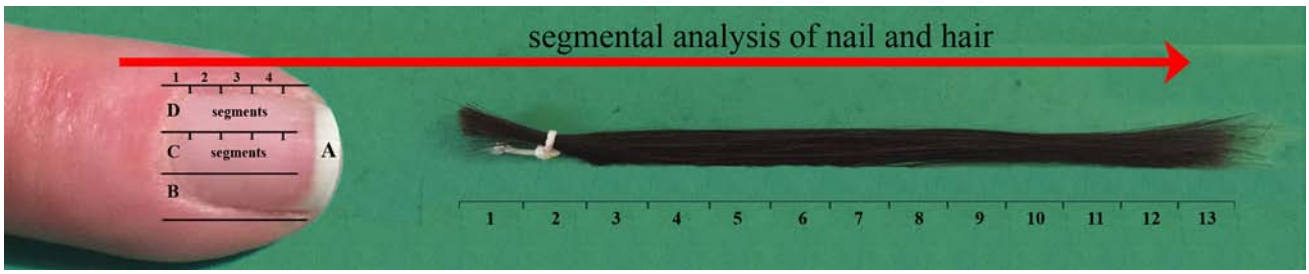

Fig. 1 Segmentation of nail and hair sample in detail. Segmentation of the whole nail samples: *A* (nail clipping), *B* (only the bottom of the nail plate), *C* (segments of nail plate excluding the *bottom*), *D* (segments of the complete nail plate). The segmentation of the hair samples were done in varying length (cm)

Table 1 presents all substances with the main transitions, internal standards and retention times for each respective method. The application of a dynamic detection mode (dMRM) provided further optimization of sensitivity, reproducibility and precision of 116 mass transitions for the psychotropic drug method, with a maximum of 26 concurrent transitions and a minimum dwell time of 17.65 ms at a minimum cycle time of 351.0 ms. In addition, there were 210 mass transitions in total for the drugs of abuse, with a maximum of 36 concurrent transitions and a minimum dwell time of 10.86 ms at a minimum cycle time of 517.0 ms. Further information about the transitions, retention times, and internal standards are given in Table 1.

Method validation

The method validation was performed according to the guidelines of the German Society of Forensic Toxicology (GTFCh) [27, 28]. Firstly, the selectivity was tested. The capability of the methods to simultaneously detect and identify the target substances, without any interferences, was confirmed by analyzing six blank nail and hair samples, once with and once without the addition of the internal standard mixture. For the calibration, 20 mg of hair or nail samples from each matrix were spiked with concentrations ranging from 0.005 to 12.5 ng/mg. The calibration range varied for the different analytes. For each calibration level ($n = 8$), six separately prepared samples were analyzed. Details are shown in Table 2. The area ratios of analyte versus internal standard were plotted to a function of drug concentration. The limit of detection (LOD) and lower limit of quantification (LLOQ) were estimated according to DIN 32645 (German standard specification) [29]. The results for each of the target substances are shown in Table 2. The accuracy of the method was confirmed daily over a period of 5 days by analyzing blank hair and nail matrix samples, which had been spiked with concentrations ranging from 0.04 to 1.5 ng/mg. The bias, repeatability, and intermediate precision were estimated. The stability of the analytes in the hair and nail samples that had been stored in the auto sampler tray for 72 h were also tested with the spiked hair and nail extracts. For each matrix, six samples at concentrations of between 0.04 and 1.5 ng/mg were prepared, pooled and divided into six aliquots, and then the peak areas were measured.

Application to real samples

The hair and nail samples from the postmortem cases ($n = 7$) were analyzed using the validated methods described above. The following cases were selected due to their case history. The first case presents a male who was treated with methadone due to opiate withdrawal and known heart disease. The second case was selected because of known drug abuse in the past and a borderline personality. Cases 3, 4, and 5 were three males with a history of past drug abuse. Current drug addiction was expected in case 6. The last case, an elderly woman from a nursing home, was selected as it was known that the decedent had had multimedications.

Results

Selectivity, linear calibration, accuracy, stability, and matrix effects

As an indication of the selectivity of the methods used in this study, no coeluting or interfering substances were apparent in the chromatogram. The results of the linear calibration were checked for outliers using the Grubbs' test with a 99 % significance level. The homogeneity of variance was confirmed by the Cochran test. Linearity was shown by applying Mandel's *F* test. For each of the 76 analytes, linearity was confirmed over the calibration

Table 1 MRM transitions (for target and qualifier), internal standard, and retention time for all substances

Target substance	Target	Qualifier	Internal standard	Retention time (min)
3-OH-Bromazepam	332.0–78.1	332.0–315.1	Tilidine-D6	7.27
6-Monoacetylmorphine	328.2–165.0	328.2–211.0	6-MAM-D3	3.82
7-Amino-flunitrazepam	284.1–135.1	284.1–256.0	7-Aminoflunitrazepam-D7	6.01
Acetylcodeine	342.2–152.1	342.2–181.1	a-Hydroxyalprazolam-D5	6.24
Amisulpride	370.1–242.1	370.1–112.1	MDE-D6	2.34
Amphetamine	136.1–91.0	136.1–119.0	Amphetamine-D5	3.16
a-OH-Alprazolam	325.0–297.0	325.0–216.0	Cocaine-D3	9.63
Benzoylecgonine	290.1–168.1	290.1–105.0	Benzoylecgonine-D3	4.96
Bisnortilidine	246.2–155.1	246.2–91.1	Cocaine-D3	6.55
Buprenorphine	468.3–55.1	468.3–414.3	Buprenorphin-D4	9.01
Bupropion	240.1–184.1	240.1–130.1	Nortilidine-D3	3.42
Chlorpromazine	319.1–86.2	319.1–58.2	Methadone-D9	8.4
Citalopram	325.2–109.1	325.2–83.1	Fentanyl-D5	5.31
Clomipramine	315.2–86.1	315.2–58.1	Methadone-D9	9.12
Clonazepam	316.0–270.0	316.0–214.0	Clonazepam-D4	10.23
Clozapine	327.1–270.1	327.1–192.1	Nortilidine-D3	3.55
Cocaethylene	318.2–196.1	318.2–82.1	Cocaethylene-D3	7.66
Cocaine	304.2–182.1	304.2–105.0	Cocaine-D3	6.61
Codeine	300.2–128.0	300.2–165.1	Codeine-D3	3.1
Desmethyldoxepin	266.1–107.1	266.4–115.1	Fentanyl-D5	5.17
Diazepam	285.1–154.0	285.1–222.1	Diazepam-D5	11.52
Dihydrocodeine	302.0–199.0	302.0–201.0	Dihydrocodeine-D6	2.99
Diphenhydramine	256.2–167.1	256.2–165.1	Fentanyl-D5	8.54
Doxepin	281.0–107.1	281.0–77.1	Fentanyl-D5	5.49
EDDP	278.2–234.1	278.2–219.1	EDDP-D3	9.22
Fentanyl	337.2–105.1	337.2–188.2	Fentanyl-D5	8.46
Flunitrazepam	314.1–268.1	314.1–239.1	Temazepam-D5	10.73
Fluoxetine	310.1–44.1	310.1–148.1	Methadone-D9	8.1
Fluphenazine	438.2–171.2	438.2–143.1	Methadone-D9	8.79
Fluvoxamine	319.1–71.1	319.1–45.1	Methadone-D9	7.00
Gabapentin	172.1–154.1	172.1–137.1	Dihydrocodeine-D6	2.89
Haloperidol	376.2–165.1	376.2–123.0	Methadone-D9	6.1
Heroin	370.2–165.1	370.2–152.1	Cocaine-D3	6.36
Hydroxyrisperidone	427.0–207.0	427.0–41.1	Nortilidine-D3	3.22
Ketamine	238.1–125.0	238.1–220.1	Ketamine-D4	4.92
Lorazepam	321.0–275.0	321.0–229.0	Lorazepam-D4	10.15
Maprotiline	278.2–191.1	278.2–189.1	Methadone-D9	7.42
MDA	180.1–163.0	180.1–135.0	MDE-D6	3.53
MDEA	208.1–163.0	208.1–105.0	MDE-D6	4.55
MDMA	194.1–163.0	194.1–105.0	MDMA-D5	3.9
Melperone	264.4–123.1	264.4–165.1	Nortilidine-D3	3.51
Methadone	310.2–265.1	310.2–77.0	Methadone-D9	10.01
Methamphetamine	150.1–91.0	150.1–119.0	Methamphetamin-D5	3.6
Methylecgonine	200.1–182.1	200.1–82.1	Methylecgonine-D3	0.86
Methylphenidate	234.1–84.1	243.1–56.1	Ketamine-D4	2.8
Metoprolol	268.2–191.2	268.2–176.1	Tramadol-D3	5.85
Mianserin	265.2–58.1	265.2–208.2	Fentanyl-D5	4.95
Midazolam	326.1–291.1	326.1–222.1	Fentanyl-D5	8.41

Table 1 continued

Target substance	Target	Qualifier	Internal standard	Retention time (min)
Mirtazapine	266.2–195.1	266.2–72.1	Tramadol-D3	2.57
Morphine	286.2–165.1	286.2–157.1	Morphine-D3	1.52
N-Desmethylcitalopram	311.2–109.1	311.2–83.1	Fentanyl-D5	5.02
N-Desmethylclomipramine	301.1–72.1	301.1–44.1	Methadone-D9	8.7
Norbuprenorphine	414.2–101.1	414.3–55.1	Norbuprenorphine-D3	7.48
Norclozapine	313.1–192.1	313.1–270.1	Nortilidine-D3	3.15
Norcocaine	290.1–168.1	290.1–77.1	Norcocaine-D3	6.83
Nordazepam	271.1–140.0	271.1–208.1	Nordiazepam-D5	10.16
Norfentanyl	233.2–84.1	233.2–150.1	Ketamine-D4	4.99
Nortilidine	260.2–155.1	260.2–128.1	Nortilidine-D3	6.72
Nortriptyline	264.2–91.1	264.2–105.1	Methadone-D9	7.13
Olanzapine	313.2–256.1	313.2–84.1	Dihydrocodeine-D6	1.84
Opipramol	364.5–171.2	364.5–143.1	Fentanyl-D5	4.16
Oxazepam	287.0–241.0	287.0–104.0	Oxazepam-D5	9.84
Oxycodone	316.2–298.1	316.2–241.1	Oxycodon-D3	3.6
Paroxetine	330.2–70.1	330.2–44.1	Methadone-D9	6.6
Promethazine	285.1–86.2	285.1–71.1	EDDP-D3	6.12
Quetiapine	384.2–253.1	384.2–221.2	Cocaethylene-D3	4.24
Risperidone	411.2–191.2	411.2–55.1	Nortilidine-D3	3.34
Sertraline	306.1–159.0	306.1–275.1	Methadone-D9	8.38
Sulpiride	342.1–112.1	342.2–214.1	Methamphetamine-D5	1.55
Temazepam	301.1–255.1	301.1–283.1	Temazepam-D5	10.82
Tilidine	274.2–155.1	274.2–153.1	Tilidine-D6	6.97
Tramadol	264.2–58.1	264.2–42.1	Tramadol-D3	5.82
Tranylcypromine	134.2–117.1	134.2–65.1	Methamphetamine-D5	1.93
Trimipramine	295.2–100.2	295.2–58.1	Methadone-D9	7.83
Venlafaxine	278.2–58.1	278.2–121.1	Nortilidine-D3	3.51
Zolpidem	308.2–65.1	308.2–236.3	Tilidine-D6	7.18

Details of main transitions, internal standards, and retention times were shown for all analytes

EDDP 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, *MDMA* 3,4-methylenedioxymethamphetamine, *MDA* 3,4-methylenedioxy-amphetamine, *MDEA* 3,4-methylenedioxy-*N*-ethyl-amphetamine, *OH* hydroxy

range. The correlation coefficients ranged between 0.9918 and 1.000 (Table 2).

The LLOQ values ranged between 1.23 and 98.6 pg/mg for hair samples and between 0.95 and 46.7 pg/mg for nail samples. In addition to this, accuracy tests were within the acceptable range as proposed in the guidelines of the GTFCh [28], presented in Table 2. Slight substance loss was observed over 72 h, which is the general period for laboratory analysis. For each analyte in both types of matrices, the maximal loss of peak area was below 25 %, which is within the acceptable range. The matrix effects in the hair and nail matrix samples were found to range from 75 to 125 %, which is within an acceptable range according to the GTFCh guidelines [28]. In addition, for all analytes in both types of matrices, the recovery was estimated to be over 50 %.

Application to real samples: exemplary case reports

Case 1

A 37-year-old male was found dead in his flat by his partner. He had been working there while renovating his apartment. The man had taken methadone to treat opiate withdrawal several years prior. He had suffered from a cardiac infarction in 2012 and had not consumed alcohol in the past. Based on the autopsy findings, he was found to have suffered from a recurrent myocardial infarction. Toxicological analysis of the femoral venous blood by HPLC-DAD [30] revealed 2.3 μg/ml of methadone and 0.1 μg/ml of metoprolol. The results from the hair and nail analyses are shown in Table 3. The hair analysis detected constant methadone and metoprolol consumption in the

Table 2 Validation parameters of 76 substances in nail and hair

Target substance	Nail						
	Linear range (ng/mg)	Weighting factor for calibration line	Correlation coefficient	LLOQ (pg/mg)	LOD (pg/mg)	Repeatability (RSD %)	Intermediate precision (RSD %)
3-OH-Bromazepam	0.25–12.5	$1/x$	0.9957	7.776	2.064	1.70	2.17
6-Monoacetylmorphine	0.25–1.25	$1/x$	0.9995	2.196	1.009	0.92	0.72
7-Amino-flunitrazepam	0.05–1.25	$1/x$	0.9995	6.125	1.404	0.56	2.01
Acetylcodeine	0.025–12.5	–	0.9985	12.52	5.202	0.98	1.02
Amisulpride	0.005–5	$1/x^2$	0.9999	1.323	1.121	2.70	3.46
Amphetamine	0.025–12.5	$1/x^2$	0.9997	9.538	3.501	1.08	1.08
a-OH-Alprazolam	0.125–12.5	$1/x^2$	0.9998	12.179	8.109	1.03	1.44
Benzoylecgonine	0.025–2.5	$1/x^2$	10.000	9.637	6.641	1.13	1.59
Bisnortilidine	0.025–12.5	$1/x^2$	0.9931	6.540	2.400	1.12	1.74
Buprenorphine	0.025–12.5	$1/x^2$	0.9998	8.690	3.401	1.18	1.29
Bupropion	0.005–5	$1/x^2$	0.9999	1.608	0.681	2.20	1.20
Chlorpromazine	0.005–5	$1/x^2$	0.9985	3.178	1.710	2.30	2.35
Citalopram	0.005–5	$1/x^2$	0.9994	1.331	1.119	3.50	3.54
Clomipramine	0.005–5	$1/x^2$	0.9983	2.466	0.744	1.50	2.52
Clonazepam	0.05–2.5	$1/x^2$	10.000	38.36	5.902	0.81	1.01
Clozapine	0.005–5	–	0.9996	1.211	0.635	3.70	3.65
Cocaethylene	0.025–12.5	$1/x^2$	0.9998	6.378	3.972	1.35	1.38
Cocaine	0.025–12.5	$1/x^2$	10.000	7.224	4.403	1.46	2.29
Codeine	0.025–5.0	$1/x^2$	0.9994	8.170	2.374	2.63	2.63
Desmethyldoxepin	0.005–5	$1/x^2$	0.9996	2.229	0.878	3.20	5.02
Diazepam	0.025–12.5	$1/x$	0.9998	7.385	4.823	0.41	1.18
Dihydrocodeine	0.025–2.5	$1/x^2$	0.9998	5.541	3.618	1.43	1.86
Diphenhydramin	0.025–12.5	–	0.9997	9.195	5.245	1.21	2.19
Doxepin	0.005–5	$1/x^2$	0.9998	1.208	1.208	1.80	4.99
EDDP	0.025–12.5	$1/x^2$	0.9998	5.286	4.501	1.53	2.91
Fentanyl	0.025–12.5	$1/x^2$	0.9999	7.818	1.804	0.98	1.72
Flunitrazepam	0.05–12.5	$1/x^2$	0.9995	15.45	5.708	1.01	2.19
Fluoxetine	0.005–5	$1/x^2$	0.9997	3.091	1.368	3.29	5.91
Fluphenazine	0.005–5	$1/x^2$	0.9974	2.848	0.910	3.20	3.19
Fluvoxamin	0.005–5	$1/x^2$	0.9999	1.899	0.554	8.33	8.33
Gabapentin	0.025–12.5	$1/x^2$	0.9987	11.88	5.917	2.03	2.03
Haloperidol	0.005–5	$1/x^2$	0.9998	2.526	0.610	2.90	2.93
Heroin	0.025–12.5	$1/x^2$	0.9998	4.011	3.440	1.79	1.97
Hydroxyrisperidone	0.005–5	$1/x^2$	0.9999	2.314	1.428	3.52	5.46
Ketamine	0.025–1.25	$1/x^2$	0.9999	6.644	4.126	0.77	2.39
Lorazepam	0.125–12.5	$1/x$	0.9993	6.820	5.326	3.98	6.17
Maprotiline	0.005–5	$1/x^2$	0.9991	2.775	2.469	3.30	3.67
MDA	0.025–12.5	$1/x^2$	0.9979	9.206	6.046	2.40	2.40
MDEA	0.025–12.5	$1/x^2$	0.9993	3.963	2.403	1.49	1.49
MDMA	0.025–12.5	$1/x^2$	0.9999	9.057	5.375	1.22	3.23
Melperone	0.005–5	$1/x^2$	10.000	2.855	0.531	2.70	2.67
Methadone	0.025–12.5	$1/x^2$	0.9989	6.585	3.822	1.82	2.55
Methamphetamine	0.025–12.5	$1/x^2$	0.9992	7.586	3.062	2.22	3.16
Methylecgonine	0.025–12.5	$1/x$	0.9996	11.766	9.623	2.60	3.45
Methylphenidat	0.005–5	$1/x^2$	0.9998	2.322	1.095	2.10	2.89
Metoprolol	0.025–2.5	$1/x$	0.9997	9.185	2.275	1.68	2.58

Table 2 continued

Target substance	Nail						
	Linear range (ng/mg)	Weighting factor for calibration line	Correlation coefficient	LLOQ (pg/mg)	LOD (pg/mg)	Repeatability (RSD %)	Intermediate precision (RSD %)
Mianserin	0.005–5	–	0.9986	2.489	0.677	5.90	6.24
Midazolam	0.025–1.25	$1/x$	0.9998	9.303	2.906	1.50	2.38
Mirtazapine	0.005–5	$1/x^2$	10.000	2.103	2.103	2.90	4.32
Morphine	0.025–12. 5	$1/x^2$	0.9999	44.66	20.27	1.20	2.04
N-Desmethylcitalopram	0.005–5	$1/x^2$	10.000	1.077	0.371	3.70	3.75
N-Desmethylclomipramine	0.005–5	$1/x^2$	0.9961	1.458	1.155	2.50	3.31
Norbuprenorphin	0.025–5.0	$1/x^2$	0.9999	12.38	1.999	1.50	1.50
Norclozapine	0.005–5	$1/x^2$	0.9997	1.777	0.666	3.40	4.92
Norcocaine	0.025–2.5	$1/x^2$	0.9999	12.67	6.553	1.40	2.28
Nordazepam	0.025–2.5	$1/x$	0.9999	7.226	4.866	1.50	2.01
Norfentanyl	0.025–5.0	$1/x^2$	0.9918	21.79	2.278	1.80	2.04
Nortilidine	0.025–12.5	$1/x^2$	0.9993	13.96	5.005	1.0	1.56
Nortriptyline	0.005–5	$1/x^2$	0.9990	2.718	0.655	3.30	4.15
Olanzapine	0.005–5	$1/x^2$	0.9997	1.911	0.426	5.80	5.84
Opipramol	0.005–5	–	0.9998	3.202	0.831	1.20	3.34
Oxazepam	0.125–12.5	$1/x^3$	0.9999	46.70	29.66	2.90	2.99
Oxycodone	0.025–12.5	$1/x$	0.9999	15.05	2.845	1.50	2.47
Paroxetine	0.005–5	$1/x^2$	0.9989	1.084	0.727	4.50	6.58
Promethazine	0.005–5	$1/x$	0.9998	2.188	0.962	2.90	3.68
Quetiapine	0.005–5	$1/x^2$	0.9999	1.525	0.629	2.90	3.32
Risperidon	0.005–5	$1/x^2$	0.9999	2.115	0.816	4.20	4.45
Sertraline	0.005–5	$1/x$	0.9982	2.529	2.529	2.00	4.92
Sulpiride	0.005–5	$1/x^2$	0.9999	1.823	1.823	3.60	4.06
Temazepam	0.025–2.5	$1/x^2$	0.9998	7.875	5.080	0.70	1.80
Tilidine	0.025–12.5	$1/x$	0.9996	9.342	7.182	1.20	2.60
Tramadol	0.025–5.0	$1/x^2$	0.9988	10.18	1.426	1.50	2.09
Tranylcypromine	0.005–5	$1/x^2$	0.9979	3.132	1.912	4.00	4.03
Trimipramine	0.005–5	$1/x^2$	0.9998	0.950	0.366	3.60	3.71
Venlafaxine	0.005–5	$1/x^2$	10.000	2.030	1.420	2.20	2.52
Zolpidem	0.025–2.5	$1/x^2$	0.9997	9.697	4.255	0.50	2.96
Target substance	Hair						
	Linear range (ng/mg)	Weighting factor for calibration line	Correlation coefficient	LLOQ (pg/mg)	LOD (pg/mg)	Repeatability (RSD %)	Intermediate precision (RSD %)
3-OH-Bromazepam	0.125–12.5	$1/x^2$	0.9995	98.63	16.04	2.50	5.92
6-Monoacetylmorphine	0.25–5.0	$1/x^2$	10.000	14.13	2.970	2.70	6.27
7-Amino-flunitrazepam	0.025–5.0	$1/x$	0.9994	14.01	6.601	1.30	4.23
Acetylcodeine	0.025–12.5	$1/x^2$	0.9997	13.33	6.322	2.40	4.99
Amisulpride	0.005–5	$1/x^2$	0.9986	91.03	40.56	2.22	3.92
Amphetamine	0.025–12.5	$1/x^2$	0.9996	9.929	5.162	2.60	2.08
a-OH-Alprazolam	0.125–12.5	$1/x^2$	10.000	8.561	4.824	2.20	4.26
Benzoylecgonine	0.025–12.5	$1/x^2$	0.9999	11.02	3.943	2.40	4.87
Bisnortilidine	0.025–5.0	$1/x^2$	0.9998	12.25	5.397	1.80	7.62
Buprenorphine	0.025–12.5	$1/x^2$	0.9999	20.09	2.040	2.30	5.20
Bupropion	0.005–5	$1/x^2$	0.9937	3.677	2.084	4.68	2.11
Chlorpromazine	0.005–5	$1/x^2$	0.9992	2.776	1.645	1.85	5.85
Citalopram	0.005–5	$1/x^2$	0.9993	3.108	1.100	4.00	4.59

Table 2 continued

Target substance	Hair						
	Linear range (ng/mg)	Weighting factor for calibration line	Correlation coefficient	LLOQ (pg/mg)	LOD (pg/mg)	Repeatability (RSD %)	Intermediate precision (RSD %)
Clomipramine	0.005–5	$1/x^2$	0.9994	6.443	1.794	3.90	4.23
Clonazepam	0.05–1.25	$1/x^2$	0.9999	49.72	22.62	1.80	5.42
Clozapine	0.005–5	$1/x^2$	0.9997	2.719	1.749	1.60	3.90
Cocaethylene	0.025–5.0	$1/x^2$	0.9999	13.91	7.22	0.40	5.80
Cocaine	0.025–12. 5	$1/x^2$	0.9999	17.63	6.91	1.10	4.05
Codeine	0.025–2.5	$1/x^2$	0.9985	5.625	5.625	2.10	3.28
Desmethyldoxepin	0.005–5	$1/x^2$	0.9997	2.580	0.496	2.0	4.47
Diazepam	0.025–12.5	$1/x^2$	0.9999	7.294	5.485	2.60	4.18
Dihydrocodeine	0.025–12.5	$1/x^2$	0.9998	16.23	9.083	2.60	3.35
Diphenhydramin	0.025–2.5	$1/x$	0.9999	17.01	8.098	1.90	6.81
Doxepin	0.005–5	$1/x$	0.9995	4.811	2.310	2.50	4.68
EDDP	0.025–12.5	$1/x^2$	0.9999	8.543	3.143	1.0	3.44
Fentanyl	0.025–12.5	$1/x^2$	0.9999	12.81	3.923	1.40	2.51
Flunitrazepam	0.025–12.5	$1/x$	0.9999	19.79	5.797	1.70	2.39
Fluoxetine	0.005–5	$1/x^2$	0.9992	2.331	0.4594	4.0	4.82
Fluphenazine	0.005–5	$1/x^2$	0.9991	3.473	1.056	5.20	5.17
Fluvoxamin	0.005–5	$1/x^2$	0.9993	3.223	1.756	2.01	7.84
Gabapentin	0.025–12.5	$1/x^2$	0.9989	12.80	3.250	2.40	4.89
Haloperidol	0.005–5	$1/x^2$	0.9994	4.200	1.684	5.85	2.46
Heroin	0.025–12.5	$1/x^2$	0.9996	12.78	4.797	1.0	2.72
Hydroxyrisperidone	0.005–5	$1/x^2$	0.9991	1.524	1.524	2.72	8.07
Ketamine	0.025–1.25	–	0.9999	18.43	2.464	2.60	1.98
Lorazepam	0.025–2.5	$1/x^2$	0.9996	38.82	33.56	2.40	4.09
Maprotiline	0.005–5	$1/x^2$	0.9991	2.202	1.224	3.90	2.40
MDA	0.025–5.0	$1/x^2$	0.9996	14.46	20.23	1.80	3.30
MDEA	0.025–5.0	$1/x^2$	0.9999	13.42	5.549	1.60	2.40
MDMA	0.025–5.0	$1/x^2$	0.9999	18.35	3.509	2.40	2.21
Melperone	0.005–5	$1/x^2$	0.9996	9.074	1.997	1.50	2.34
Methadone	0.025–5.0	$1/x^2$	0.9999	16.10	8.221	1.10	2.93
Methamphetamine	0.025–12.5	$1/x^2$	0.9999	13.78	7.285	1.80	2.38
Methylecgonine	0.025–5.0	$1/x$	0.9998	18.93	16.74	2.30	4.46
Methylphenidat	0.005–5	$1/x^2$	0.9978	2.479	0.719	3.10	2.12
Metoprolol	0.025–5.0	$1/x^2$	0.9995	12.07	3.736	1.70	2.64
Mianserin	0.005–5	$1/x^2$	0.9994	6.836	1.793	3.30	2.57
Midazolam	0.025–5.0	$1/x^2$	0.9998	10.46	6.033	1.40	2.71
Mirtazapine	0.005–5	$1/x^2$	0.9999	6.49	2.36	5.80	2.90
Morphine	0.025–5.0	$1/x^2$	0.9999	44.44	16.26	1.50	1.45
N-Desmethylcitalopram	0.005–5	$1/x^2$	0.9998	2.798	1.055	1.30	2.50
N-Desmethylclomipramine	0.005–5	$1/x^2$	0.9947	2.164	1.022	3.30	2.16
Norbuprenorphin	0.025–5.0	$1/x^2$	0.9999	17.54	6.669	2.60	4.26
Norclozapine	0.005–5	$1/x^2$	0.9995	2.087	0.856	1.70	2.54
Norcocaine	0.025–5.0	$1/x^2$	0.9999	11.41	5.890	2.30	2.71
Nordazepam	0.025–12.5	–	0.9999	16.68	3.756	2.10	3.47
Norfentanyl	0.025–2.5	$1/x^2$	0.9999	17.85	1.840	2.50	3.16
Nortilidine	0.025–5.0	–	0.9999	17.77	3.476	3.0	2.63
Nortriptyline	0.005–5	$1/x^2$	0.9991	1.234	1.095	3.20	2.79

Table 2 continued

Target substance	Hair						
	Linear range (ng/mg)	Weighting factor for calibration line	Correlation coefficient	LLOQ (pg/mg)	LOD (pg/mg)	Repeatability (RSD %)	Intermediate precision (RSD %)
Olanzapine	0.005–5	$1/x^2$	0.9938	8.86	1.12	4.08	2.89
Opipramol	0.005–5	$1/x^2$	0.9997	10.51	5.954	3.40	2.24
Oxazepam	0.125–5.0	$1/x^3$	0.9999	45.53	37.03	2.60	3.21
Oxycodone	0.025–12.5	$1/x^2$	0.9998	17.94	3.684	1.70	3.61
Paroxetine	0.005–5	$1/x^2$	0.9997	5.980	2.517	3.40	2.74
Promethazine	0.005–5	$1/x^2$	0.9997	2.617	0.670	4.40	2.59
Quetiapine	0.005–5	$1/x^2$	0.9997	1.572	0.667	3.04	3.73
Risperidon	0.005–5	$1/x$	0.9999	2.078	0.778	2.38	2.37
Sertraline	0.005–5	$1/x^2$	0.9996	9.120	4.510	2.40	2.14
Sulpiride	0.005–5	–	0.9988	1.912	0.283	4.10	3.23
Temazepam	0.025–12.5	$1/x^2$	0.9999	10.79	5.118	2.20	3.03
Tilidine	0.025–5.0	$1/x^2$	0.9998	13.73	3.238	1.20	2.71
Tramadol	0.025–5.0	$1/x^2$	0.9997	22.15	3.675	1.20	3.40
Tranylcypromine	0.005–5	$1/x$	0.9999	3.548	1.522	4.57	2.20
Trimipramine	0.005–5	$1/x^2$	0.9988	3.716	1.487	7.66	3.86
Venlafaxine	0.005–5	$1/x^2$	0.9996	1.380	0.394	4.30	1.88
Zolpidem	0.025–5.0	$1/x^2$	0.9999	6.567	4.579	1.0	1.20

In this table validation results were displayed for all 76 analytes in nail and hair

LLOQ lower limit of quantification, *LOD* limit of detection, *RSD* relative standard deviation, *OH* hydroxy, *EDDP* 2-ethylidene-1.5-dimethyl-3.3-diphenylpyrrolidine, *MDA* 3.4-methylenedioxy-amphetamine, *MDEA* 3.4-methylenedioxy-*N*-ethyl-amphetamine, *MDMA* 3.4-methylenedioxymethamphetamine

months prior to death. The measured methadone concentrations were within the typical ranges detected in hair samples of people in methadone treatment program [2]. In hair segment 3 (4–6 cm) we detected the presence of amphetamine. Minor contact with cocaine was observed from the analysis of hair segments 2, 3, and 4. In contrast, amphetamine was only detected in nail segment C4. The nail analysis showed lower concentrations of benzoylecgonine than the hair segments, and cocaine was not detected in any of the nail segments. The segmental toenail analysis showed constant consumption of methadone and metoprolol. In contrast to the segmental analysis of the hair sample, heroin metabolites 6-monoacetylmorphine, morphine, and codeine were detected in nail segment C3 (4–6 mm) and in the nail clipping. Concentrations in the nail clipping were approximately 10 % of the concentration found in segment C3. In contrast, higher drug concentrations were found in the nail clipping compared to the nail segments in other cases, in which drug intake (close to the time of death) was able to be confirmed by blood analysis. The concentration ratio of heroin metabolites, 6-monoacetylmorphine and morphine, were found to differ, with 0.15 detected in the nail clipping and 0.34 in nail segment C3. In addition to this, the EDDP/methadone concentration ratios were similar, with 0.07 detected in the nail clipping, 0.09 in the nail segments (values ranged from 0.05 to 0.10 with a mean of 0.07 for all nail segments) and 0.09 in the hair segments (values ranged from 0.06 to 0.09 with a mean of 0.07). The mean concentration ratios of EDDP/methadone in the hair and nail segments were similar.

Case 2

A 24-year-old male was found dead in his bathroom by the concierge. His mother had contacted the concierge because she was worried about her son. The deceased suffered from borderline personality disorder and was addicted to several different drugs of abuse. Intoxication at the time of death was assumed from the scene and the autopsy findings. Toxicological analysis of the femoral venous blood by HPLC-DAD revealed 0.2 µg/ml of diazepam and 0.3 µg/ml of nordazepam [30]. The urine sample tested positive for 6-monoacetylmorphine, morphine, codeine, papaverine, noscapine, diazepam, nordazepam, zolpidem, ibuprofen, caffeine, and nicotine by LC-MS/MS. A combined intoxication with heroin and benzodiazepines was assumed. The results from the hair and nail analyses are shown in

Table 3 Segmental nail and hair analyses from case 1

Target substance	Concentration in nail segments (ng/mg)									Concentration in hair segments (ng/mg)			
	A 13–15 mm	B 0–15 mm	C1 0–2 mm	C2 2–4 mm	C3 4–6 mm	C4 6–8 mm	D1 0–2 mm	D2 2–4 mm	D3 4–6 mm	D4 6–8 mm	1 0–2 cm	2 2–4 cm	3 4–6 cm	4 6–8 cm
6-Monoacetylmorphine	0.010	n.d.	n.d.	n.d.	0.159	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Amphetamine	n.d.	n.d.	n.d.	n.d.	n.d.	0.008	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.011	n.d.
Benzoylecgonine	0.015	n.d.	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	n.d.	0.042	0.043	0.050
Cocaine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.02	0.02	0.03
Codeine	n.d.	n.d.	n.d.	n.d.	0.055	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EDDP	1.61	0.789	1.09	1.72	1.05	1.42	1.13	0.819	1.07	1.39	2.29	3.04	3.24	3.53
Methadone	21.1	15.8	20.5	18.0	11.1	15.1	20.4	11.6	12.0	13.1	33.30	50.32	32.89	44.23
Metoprolol	4.02	1.35	1.32	1.29	1.19	1.79	1.63	1.12	1.64	2.05	4.18	5.24	3.64	6.65
Morphine	0.064	n.d.	n.d.	n.d.	0.460	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Results of the segmental toenail and hair analyses from a 37-year-old man

LLOQ lower limit of quantification, *n.d.* not detected

Table 4. Constant consumption of heroin, cocaine, clonazepam, benzodiazepines, doxepin, tramadol, and zolpidem was determined by the segmental hair and nail analyses. The concentrations of zolpidem and clonazepam in hair showed consistently high intake in the 8 months prior to death. Furthermore, sporadic consumption of diazepam, doxepin, and tramadol was determined by the segmental analysis of the hair bundle.

The segmentation of the toenail also showed varied concentrations in each of the different segments for the analytes diazepam, doxepin, and tramadol. Consistent with the increasing concentration of 6-monoacetylmorphine and morphine in hair segments 1–4, the nail segments D1–D4 also showed increasing concentrations of the heroin metabolites 6-monoacetylmorphine and morphine. Decreasing intake of heroin and cocaine in the month prior to death was determined by the hair analysis from a decreasing concentration in the different hair segments. The segments D1 to D4 also included the underneath of the nail matrix, which is in direct contact with the nail bed. High concentrations of 6-monoacetylmorphine, morphine, codeine, benzoylecgonine, tramadol, and doxepin were detected in the nail clipping. The heroin metabolites, 6-monoacetylmorphine and morphine, had concentrations ratios of 0.09 in the nail clipping and ranged between 0.001 and 0.10 in the nail segment. In this case, the concentration ratios in the corresponding hair segments ranged from 0.5 to 10.6. Furthermore, nordazepam to diazepam were also found in concentration ratios of 2.99 in the nail clipping, 1.55–2.12 in the nail segments and 0.40–1.04 in the hair segments. The temazepam/diazepam concentration ratios were calculated to be 0.11 in the nail clipping, 0.13–0.26 in the nail segments and 0.05–0.08 in the hair segments.

Case 3

A 70-year old male was found in a state of putrifaction in his flat, and had last been seen alive a week prior. He was disabled and had home care. The police found a large amount of different drugs (including tramadol, tilidine, diazepam, amitriptyline, omeprazole, acetylsalicylic acid, diclofenac, enalapril, and nebivolol) in his flat, and therefore suspected a drug addiction. No cause of death could be identified based on the autopsy findings. The toxicology findings in the liver tissue, analyzed by HPLC-DAD, revealed 65 ng/g of diazepam and 232 ng/g of nordazepam, but based on these findings intoxication could not be determined [30].

The results from the hair and nail analyses are shown in Table 5. In the hair and nail segments nortilidine, bisnortilidine, diazepam, nordazepam, temazepam, oxazepam, and tramadol were identified. In addition, methylphenidate was detected in the nail. The concentration of analytes in

Table 4 Segmental toenail and hair analyses from case 2

Target substance	Concentration in nail segments (ng/mg)										Concentration in hair segments (ng/mg)			
	A 13–18 mm	B 0–18 mm	C1 0–2 mm	C2 2–4 mm	C3 4–6 mm	C4 6–8 mm	D1 0–2 mm	D2 2–4 mm	D3 4–6 mm	D4 6–8 mm	1 0–2 cm	2 2–4 cm	3 4–6 cm	4 6–7.5 cm
6-Monoacetylmorphine	3.92	n.d.	n.d.	0.006	0.016	0.035	0.026	0.057	0.144	1.68	0.042	0.140	0.432	1.692
Acetylcodeine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.013	0.029	0.104
Benzoylecgonine	0.467	0.057	0.087	0.078	0.098	0.077	0.115	0.123	0.117	0.169	0.042	0.141	0.232	0.803
Clonazepam	<LLOQ	n.d.	0.157	0.173	0.133	0.035	0.138	0.079	0.074	0.056	0.319	0.172	0.233	0.328
Cocaethylene	<LLOQ	n.d.	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	0.011	0.206
Cocaine	<LLOQ	n.d.	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	0.044	0.209	0.527	1.16
Codeine	3.72	0.220	0.259	0.270	0.293	0.290	0.557	0.456	0.587	1.18	0.020	0.261	0.086	0.106
Diazepam	0.241	0.639	0.374	0.356	0.272	0.101	0.364	0.434	0.410	0.380	0.402	0.464	0.604	0.810
Heroin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.829	0.831	0.198	0.900
Doxepin	0.690	0.098	0.209	0.356	0.472	0.139	0.155	0.209	0.159	0.257	n.d.	n.d.	0.060	0.211
Morphine	42.8	3.14	3.03	3.14	3.26	3.37	6.68	5.85	5.03	13.01	0.084	0.095	0.075	0.159
Norcocaine	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	n.d.	n.d.	<LLOQ	0.025
Nordazepam	0.721	1.08	0.580	0.645	0.608	0.234	0.608	0.921	0.812	0.745	0.422	0.374	0.247	0.433
Oxazepam	<LLOQ	0.068	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	0.041	<LLOQ	<LLOQ	0.020	0.026	0.012	0.014
Temazepam	0.028	0.168	0.064	0.056	0.043	0.014	0.062	0.078	0.074	0.060	0.036	0.027	0.032	0.052
Tramadol	0.358	0.051	0.056	0.100	0.160	0.162	0.102	0.127	0.258	0.341	0.023	0.821	0.148	0.121
Zolpidem	0.045	0.025	0.021	0.018	0.015	0.010	0.016	0.018	0.017	0.017	0.113	0.143	0.127	0.100

Results of the segmental toenail and hair analyses from a 24-year-old man

LLOQ lower limit of quantification, *n.d.* not detected

the nail clipping (17–19 mm) were consistent with the concentration determined in the other segments 2–19 mm (without a free edge) and in the whole nail sample 0–19 mm which included the free edge.

Higher concentrations of nortilidine and bisnortilidine were detected in the hair segment 4–5.5 cm compared to the hair segment 2–4 cm and in the whole hair strand 0–5.5 cm. Similar concentrations of diazepam and the metabolites nordazepam, temazepam, and oxazepam were measured in each of the hair segments. Nordazepam, temazepam, and oxazepam were also present in the seizures as their single and separated drug forms. Furthermore, the concentration ratios of bisnortilidine/nortilidine were 0.83 in the nail clipping, 0.63 in the nail segment and in whole nail 0.78 was detected. In the hair samples, concentration ratios of 1.11 (2–4 cm), 0.76 (4–5.5 cm), and 1.50 (0–5.5) were estimated. A mean concentration ratio of 0.74 in nail and 1.10 in hair was observed for bisnortilidine/nortilidine. For nordazepam/diazepam, 3.33 was detected in the nail clipping, 2.63 in the nail segment (2–19 mm), and 2.23 in the whole nail sample. In addition, the concentration ratios of nordazepam/diazepam were calculated to be 1.01 (2–4 cm), 1.05 (4–5.5 cm), and 1.31 (0–5.5 cm) in the hair segments. Based on these data, the concentration ratios of temazepam/diazepam were estimated to be 0.29 in the nail clipping, 0.14 (2–19 mm) in the nail segment and 0.49 in the whole nail. In the hair, the concentration ratios were calculated to be 0.04 (2–4 cm), 0.08 (4–5.5 cm), and 0.06 (0–5.5 cm).

Case 4

A 63-year-old male had died in hospital after his sister found him unconscious in his flat. He had suffered from a hepatitis C virus infection, drug addiction, coagulation disorder, and liver cirrhosis. According to his statement, he had been hit, and blood was found in his mouth in addition to hematoma and excoriations all over his body. The police had found methadone in his flat and assumed that he was in a substitution therapy program with methadone. There was no clear cause of death following the autopsy. Toxicological analysis of femoral venous blood by HPLC-DAD revealed 0.05 μg/ml of doxepin, 0.17 μg/ml of desmethyldoxepin, 0.16 μg/ml of diazepam, 0.50 μg/ml of nordazepam, 0.36 μg/ml of methadone, 0.37 μg/ml of EDDP, 0.61 μg/ml of lidocaine, 0.01 μg/ml of tramadol, 0.09 μg/ml of oxazepam, 0.09 μg/ml of temazepam and 0.03 μg/ml of trimipramine [30]. Each of the drug concentrations detected were within a therapeutical range. The results from the hair and nail analyses are shown in Table 6. The hair analysis showed the presence of doxepin, trimipramine, diazepam, and methadone in the month before death. A higher concentration of the methadone metabolite EDDP and the benzodiazepine temazepam were detected in the nail clipping compared to the hair sample. The concentration ratios of desmethyldoxepin/doxepin were calculated to be 0.49 in the nail clipping and 0.85 in the hair. The concentration ratios of nordazepam/diazepam and temazepam/diazepam were similar in the nail clipping. In contrast, concentration ratios in the hair of nordazepam/diazepam (1.62) and temazepam/diazepam (0.05) were estimated. Different concentration ratios of EDDP/methadone were estimated in the nail clipping (2.35) and hair (0.08).

Case 5

A 33-year-old male was found dead in his flat. He had a history of heroin abuse, but was attempting to withdraw from heroin at the time of his death. The police found hints of consumption of other drugs, including cannabinoids and methoxamine (MXE). One of his friends had told the police that the deceased

Table 5 Segmental nail and hair analyses from case 3

Target substance	Concentration in nail segments (ng/mg)			Concentration in hair segments (ng/mg)		
	17–19 mm	2–19 mm	0–19 mm	2–4 cm	4–5.5 cm	0–5.5 cm
Methylphenidate	0.007	0.008	0.04	n.d.	n.d.	n.d.
Bisnortilidine	0.05	0.03	0.03	0.01	0.07	0.06
Diazepam	0.790	0.804	0.631	3.18	2.99	3.12
Nordazepam	2.63	2.12	1.41	3.23	3.14	4.09
Nortilidine	0.060	0.047	0.038	0.009	0.091	0.04
Oxazepam	0.099	0.061	0.062	0.064	0.100	0.020
Temazepam	0.235	0.119	0.311	0.152	0.267	0.216
Tramadol	11.6	7.35	8.72	11.1	11.8	8.80

Results of the segmental toenail and hair analyses from a 70-year-old man. The nail was separated into three segments: whole nail (0–19 mm), nail segment without the free edge (2–19 mm), and nail clipping (17–19 mm)

n.d. not detected

Table 6 Nail and hair analyses from case 4

Target substance	Concentration in nail clipping (ng/mg) 3 mm	Concentration in hair (ng/mg) 0–6 cm
Desmethyldoxepin	0.453	0.655
Doxepin	0.916	0.766
Trimipramine	0.01	0.01
Diazepam	0.148	0.207
EDDP	23.11	1.55
Methadone	9.804	18.28
Nordiazepam	0.913	0.337
Oxazepam	2.33	<LLOQ
Temazepam	1.45	0.01

Results of the nail clipping and hair analyses from a 63-year-old man

LLOQ lower limit of quantification

had used subutex (buprenorphine) and diazepam in the days before he died. The toxicological analysis of femoral venous blood revealed 0.04 μg/ml of morphine, 0.003 μg/ml of codeine, 0.001 μg/ml of norbuprenophine, 0.12 μg/ml of fentanyl, 0.01 μg/ml of norfentanyl, 0.01 μg/ml of amphetamine, 0.08 μg/ml of oxazepam, 0.31 μg/ml of nordazepam, 0.13 μg/ml of diazepam and 0.13 μg/ml of temazepam. From this, combined intoxication with opioids and benzodiazepines was suggested [30]. The urine tested positive for the analytes 6-monoacetylmorphine, codeine, morphine, methadone, EDDP (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine), buprenorphine, norbuprenorphine, nordazepam, oxazepam, and temazepam. Thus, it was assumed that the man had consumed heroin, diazepam, fentanyl, methadone, and buprenorphine several hours before death. The results of the hair and nail analyses are shown in Table 7. The hair analysis revealed the use of heroin and benzodiazepines in addition to the recreational drug use of amphetamine, buprenorphine, tramadol, ketamine, and diphenhydramin. Concerning the measured concentration in the hair sample, he had rarely abused cocaine, clonazepam, and methadone in the past.

Higher concentrations of 6-monoacetylmorphine (6-MAM), amphetamine, fentanyl, morphine, codeine, and acetylcodeine were measured in the nail clipping than in hair matrix. Heroin intake close to the time of death was confirmed based on the blood and urine results. The higher concentrations detected in the nail clipping may have been due to incorporation via sweat or external contamination. The heroin metabolites 6-monoacetylmorphine and morphine concentration ratios were calculated to be 0.59 in the nail clipping and 0.04 in hair. The concentrations ratios of EDDP/methadone were 0.10 in the nail clipping and 0.08 in hair, the ratios of nordazepam/diazepam were 0.64 in the nail clipping and 0.87 in hair, the ratios of temazepam/diazepam were 0.05 in the nail clipping and only 0.07 in hair, ratios of norbuprenorphine/buprenorphine were 0.07 in the nail clipping and 1.07 in hair and finally, the concentration ratios of norfentanyl/fentanyl were 0.01 in the nail clipping and 0.03 in hair in this case. In addition to the preparation of the hair sample using the ball mill, the hair was also cut into small pieces using scissors. In our laboratory, it is routine for hair samples to be cut into small pieces for drug detection, and not ground using the ball mill. Table 7 shows also the results using both techniques for sample preparation before the extraction. The analytes 6-MAM, acetylcodeine, cocaine, and diphenhydramine showed the highest differences between the cut and milled hair samples from this case. The cocaine metabolite benzoylecgonine was detected in a higher concentration in the hair sample after milling than in the hair sample which was cut into small pieces. The 6-MAM concentration in the hair sample using the routine process of cutting into small pieces was 6.55 ng/mg compared to 0.105 ng/mg determined in the hair sample after milling. In addition to this, the concentration of cocaine detected in the cut hair was 0.05 ng/mg, but was under the limit of quantification in the milled hair sample.

Case 6

A 36-year-old male was found in a hotel room in a state of putrefaction. The police found drug supplies (e.g., small zip lock bag and two syringes) beside the body. A statement from his mother indicated that he suffered from depression and addictions to alcohol and gambling. A fresh cardiac infarction was detected from the autopsy. Toxicological analysis of the femoral venous blood revealed 0.11 μg/ml of diazepam, 0.05 μg/ml of nordazepam, 0.04 μg/ml of morphine, 0.003 μg/ml of codeine, 0.005 μg/ml of lorazepam, and 1.78 g/kg of alcohol [30]. In addition, the hair analysis revealed the use of heroin, cocaine, MDMA, amphetamine, ketamine, and diazepam. The results of the hair and nail analyses are shown in Table 8.

Table 7 Nail and hair analyses from case 5

Target substance	Concentration in nail clipping (ng/mg) 5 mm	Concentration in hair sample after milling (ng/mg) 0–3.5 cm	Concentration in hair sample after cutting into small pieces (ng/mg) 0–3.5 cm
6-Monoacetylmorphine	9.22	0.105	6.55
Acetylcodeine	0.388	<LLOQ	0.37
Amphetamine	3.27	1.04	1.63
Benzoylecgonine	0.041	0.012	<LLOQ
Buprenorphine	0.435	0.043	0.08
Clonazepam	0.039	<LLOQ	0.06
Cocaine	0.014	<LLOQ	0.05
Codeine	1.78	0.576	0.80
Diazepam	1.68	1.40	2.50
Diphenhydramine	0.390	0.431	2.38
EDDP	0.013	0.005	n.d.
Fentanyl	1.80	0.681	0.54
Ketamine	0.031	n.d.	n.d.
Mirtazapine	0.004	<LLOQ	n.d.
Methadone	0.124	0.08	n.d.
Morphine	15.6	2.35	4.96
Norbuprenorphine	0.031	0.046	0.12
Nordazepam	1.09	1.23	1.71
Norfentanyl	0.02	0.021	n.d.
Oxazepam	0.127	0.211	0.56
Temazepam	0.082	0.110	0.19
Tramadol	0.330	0.114	0.79

Results of the nail clipping and hair analyses from a 33-year-old man. Hair sample preparation was done in two different ways: milling or only cut into small pieces before the extraction

LLOQ lower limit of quantification, *n.d.* not detected

Low concentrations of 6-monoacetylmorphine (6-MAM) in the nail segments were identified. Higher concentrations of MDMA, MDA, and diazepam were detected in the 8-11 mm nail clipping than in the 0–8 mm segment. It is possible that the drugs were incorporated via sweat close to the time of death. For nordazepam and diazepam, the concentration ratios were found to be 0.79 and 0.59 in the nail and 0.23 in the hair sample. In this case, the concentration ratios of the heroin metabolites 6-MAM to morphine were 0.02 in the nail and 0.7 in hair. The analytes MDA to MDMA showed similar concentration ratios of 0.09 and 0.07 in the nail and 0.10 in hair. Madry et al. [31] analyzed nail and hair samples after a single dose of MDMA, and reported the same ratios of MDMA to its metabolite in hair and nail samples.

Case 7

An 86-year-old female died in a nursing home. She had collapsed and there had been attempts to resuscitate her. A nurse told the police that pipamperone was given to the patients for sedation, and had also been given to the deceased female. There was no other information given regarding her medical history. A two-sided pulmonary embolism appeared to be the probable cause of death. The toxicological analysis of femoral venous blood revealed 0.24 μg/ml of pipamperone, 0.06 μg/ml of bisnortilidine, 0.04 μg/ml of nortilidine, 0.08 μg/ml of mirtazapine, 0.03 μg/ml of zolpidem, and 0.01 μg/ml of amiodarone [30]. All measured concentrations were within a therapeutic range, and therefore, the therapeutic use of the medication was assumed.

The results from the hair and nail analyses are shown in Table 9. In both the hair and toenail samples mirtazapine, tilidine, nortilidine, bisnortilidine, opipramol, and zolpidem were detected. Lower concentrations of tilidine, nortilidine, bisnortilidine, and mirtazapine were found in the hair sample. Diluted effects due to the hair length of 10 cm were possible in this case. Approximately similar concentration ratios of the metabolites bisnortilidine/nortilidine of 1.32 in the toenail and 1.56 in hair were observed.

Table 8 Segmental nail and hair analyses from case 6

Target substance	Concentrations in nail segments (ng/mg)		Concentrations in hair (ng/mg)
	8–11 mm	0–8 mm	0–8 cm
6-Monoacetylmorphine	0.004	0.01	1.76
Amphetamine	0.637	1.32	1.02
Acetylcodeine	n.d.	n.d.	0.038
Cocaine	n.d.	n.d.	0.075
Codeine	0.03	n.d.	0.207
Diazepam	0.372	0.119	0.582
Benzoylecgonine	n.d.	0.009	0.01
Ketamine	0.013	0.076	0.068
MDA	0.110	0.365	0.627
MDMA	1.41	3.81	6.06
Morphine	0.155	n.d.	2.49
Nordazepam	0.221	0.095	0.136

Results of the segmental toenail and hair analyses from a 36-year-old man. The nail was separated into two segments: whole nail (0–8 mm) and the nail clipping (8–11 mm)

n.d. not detected

Discussion

This study shows that the concentrations of the detected substances in hair and nail samples taken from postmortem cases were not comparable. Furthermore, different concentrations of drugs in the different nail segments were detected. Based on the results of case 1, in which 6-MAM, the primary heroin metabolite, was only detected in the nail segment C3 and the nail clipping, the detection of a single or rare drug intake may be possible by the segmental nail analysis. This incorporation may have occurred mainly via the blood, which was in direct contact with the nail root, and also may have occurred during the formation of the nail plate. The detection of the heroin metabolites in the nail clipping may be explained by an external contamination at the time of drug intake months before the time of death. Analytes detected in the nail clipping may also have originated from intake of the drug months before or from external contamination via sweat. The lower concentrations in nail clipping compared to the concentrations in the segment C3 may be caused by wash out effects. However, there was no evidence of acute heroin intake near to death. This was also concluded from the blood and urine analyses. The hair samples from case 1 did not detect heroin consumption in the months leading up to death. In contrast, external contamination cannot be excluded for the results obtained from the hair and nail samples in these cases, as external contamination has previously been reported in the hair matrix [32–34]. Higher concentrations of the analytes were detected in the nail clipping compared to the other nail segments, which may be explained by external contamination before intake of the drug. In addition, drug incorporation via sweat following the drug intake is another possibility for the detection of a higher concentration in the nail clippings. In general, case 2 showed higher concentrations of 6-monoacetylmorphine, codeine, morphine, benzoylecgonine, and tramadol in the nail clipping than in the nail segments. The results from the

Table 9 Nail and hair analyses from case 7

Target substance	Concentration in whole nail (ng/mg) 0–10 mm	Concentration in hair (ng/mg) 0–10 cm
Mirtazapine	0.019	<LLOQ
Bisnortilidine	0.358	0.075
Opipramol	0.023	0.092
Nortilidine	0.270	0.048
Tilidine	0.012	<LLOQ
Zolpidem	0.024	0.083

Results of the whole nail and hair analyses from a 86-year-old woman

LLOQ lower limit of quantification

urine analysis showed prior heroin intake before death. External contamination or drug incorporation via sweat are probable reasons for the higher drug concentration detected in the nail clippings compared to the nail segments.

The analytes MDA/MDMA, EDDP/methadone, and the metabolites of tilidine bisnortilidine/nortilidine had similar concentration ratios in the nail and hair samples in five of the different cases. Methadone and its metabolite EDDP were detected in cases 1, 4, and 5, shown in Tables 3, 6, and 7. In case 4, the concentration ratios of EDDP/methadone were different from cases 1 and 5. The different concentration ratios of EDDP to methadone may be caused by differences in the intensity of sweating of each individual. After methadone intake, most patients suffer from heavy perspiration [35, 36], and therefore, higher drug incorporation via the sweat was likely. All cases showed acute methadone intake near to the time of death as detected by the blood and urine analyses.

The concentration ratios of diazepam and its metabolites nordazepam and temazepam in cases 2, 3, 4, 5, and 6 were not comparable between the hair and nail samples. In the hair samples, the concentration ratios for temazepam/diazepam were higher than for the nail samples, demonstrated by analysis in the different cases. The same concentration ratios of nordazepam/diazepam and temazepam/diazepam were detected in the nail clipping and hair samples of case 5. In this study, the concentration of norbuprenorphine was only 10 % of buprenorphine in the nail clipping, and similar concentrations of norbuprenorphine and buprenorphine in hair were calculated. In contrast, Skopp et al. detected a higher concentration of norbuprenorphine (about 10 %) compared to buprenorphine in hair samples. The sample preparation procedure may be causative for the different concentration ratios [37].

In this study, the concentrations of benzoylecgonine were higher than the cocaine concentrations in the nail samples, while in hair samples the concentration of cocaine was higher (about 5–10 % [38]) than the concentration of benzoylecgonine. This may be due to the sample preparation technique used in this study. Decreased concentrations of analytes in the nail and hair samples, as a result of hydrolysis, e.g., 6-monoacetylmorphine or cocaine, is likely due to the high temperature reached during the milling of the hair and nail samples. Usually the hair samples are only cut into small pieces using scissors prior to the extraction steps (routine processing) [26]. Cases 2, 5, and 6 had different concentration ratios of 6-monoacetylmorphine to morphine in the hair and nail samples.

Table 7 shows the drug concentrations found in case 5. In this case the hair was either milled or cut into small pieces before the extraction. In general, the highest loss of drug concentration in the hair after milling was found for 6-MAM. The hydrolysis of 6-MAM to morphine during the milling process may be the reason for the inconsistent concentration ratios between the different cases in this study. In addition, for all other analytes there were only small differences. However, it is notable that the two hair samples from case 5 for the routine analysis and for use in this study were taken from different areas of the head. Because of this, differences in the drug concentration may be expected. In this case, the concentration of cocaine was very low and therefore, differences could not be clearly observed. However, the cocaine metabolite benzoylecgonine was present in a higher concentration in the hair sample after milling than in the hair sample which was routinely processed. It is possible that the analyte cocaine was hydrolyzed to benzoylecgonine during the milling of the hair sample. Further research is required to clarify the degree of hydrolysis that occurs during the milling process. In addition, the use of a refrigerated ball mill may reduce unstable analytes, which needs to be confirmed in future research.

The calculation of concentration ratios may be helpful to discriminate between external contamination and drug incorporation after drug intake (e.g., via the blood) [35]. In case 6, similar concentration ratios of MDA/MDMA in the nail clipping and nail segments were calculated. The blood analysis revealed no MDMA intake close to the time of death, and no external contamination was assumed in this case. Case 2 had a different concentration ratio in the nail clipping and nail segment for 6-MAM/morphine. In addition, the concentration of the primary heroin metabolite 6-MAM was higher in the nail clipping than in any other segment. In this case, the urine analysis revealed heroin intake several hours prior to death, suggesting that external contamination was probable. The discrimination between drug intake and external contamination may be possible by calculating the concentration ratios, however, further research is needed.

Conclusion

The main aims of this study were to validate sensitive methods for the determination of a wide variety of drugs in nail and hair samples, and to analyze real nail and hair samples from postmortem cases. The application to real samples demonstrated the usefulness of nail analysis for the long-term detection of previous drug intake for a wide range of drugs. We also showed that the concentrations of drugs in the hair and nail samples were not comparable, and the frequency of drug intake may not be concluded from the determination of the drug concentrations in nail samples as it could for the hair samples. From the segmental nail analysis, we demonstrated that different drug concentrations were incorporated in different segments.

Based on these results, drug incorporation via the germinal matrix appeared to be the main way that drugs were incorporated. Constant drug incorporation into nail following drug intake (via the blood) from the nail bed appeared to be negligible. Higher drug concentrations were detected in nail clippings than in the single nail segments, which may be due to drug intake close to the time of death. Sweat or external contamination may be mechanisms for the incorporation of drugs into the free edge of the nail. One case showed a positive result for heroin consumption several months prior to death from the detection of heroin metabolites in only one segment and also in the nail clipping. Based in this finding, an important way of drug incorporation into nail matrix, may be an incorporation via blood during the nail formation in the nail root. The small concentrations of heroin metabolites in the nail clipping may originate from external contamination months before the time of death. Certainly, wash out effects from the nail clipping must be considered in this case.

To the best of our knowledge, this is the first study to include a segmental nail analysis and to compare the segmental hair analysis from postmortem cases of individuals with prior drug use. However, further research is required to clarify the mechanism of drug incorporation into the nails, and to verify the effectiveness of these washing steps and the influence of nail polish on the drug incorporation rate.

Key points

1. Analysis of nails was confirmed as a useful retrospective technique for the detection of previous drug intake.
2. For some analytes, including MDA/MDMA, EDDP/methadone, and nortilidine/bisnortilidine, similar concentrations ratios between parent drug and main metabolite in the hair and nail samples were detected.
3. Drug incorporation into the nail root, via the germinal matrix, appeared to be the main way of incorporation. For the interpretation drug incorporation via external contamination in nail clippings should be assumed.
4. Drug concentrations in nail segments and hair segments were not comparable.

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